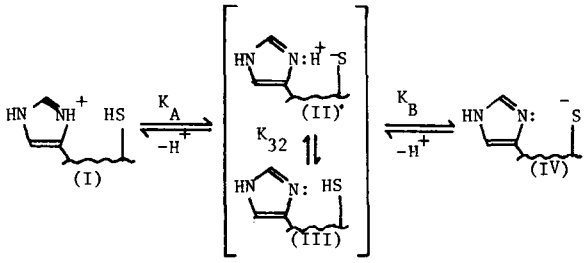
# IS THE THIOLATE–IMIDAZOLIUM ION PAIR THE CATALYTICALLY IMPORTANT FORM OF PAPAIN?

Donald J. CREIGHTON, Morris S. GESSOUROUN and James M. HEAPES
*The Laboratory for Chemical Dynamics, Department of Chemistry, University of Maryland Baltimore County, Catonsville, MD, USA*

Received 5 December 1979

## 1. Introduction

A basic step in the catalytic mechanism of papain, as well as other sulfhydryl proteases, involves nucleophilic attack of an active site sulfhydryl group on polypeptide substrates resulting in the formation of a thioacyl–enzyme intermediate [1]. The proximity of an imidazole sidechain to the active site sulfhydryl suggests that this group may play an important role in catalyzing the acylation reaction [2,3]. For the simple system of pH-dependent equilibria shown below, consistent with a bell-shaped pH-acylation rate profile, catalytic assistance could occur in either one or both of two extreme tautomeric forms (II,III) envisioned to predominate at the pH-optimum of activity (pH ~ 6.5).




The thiolate–imidazolium ion pair (II) has been concluded to be the catalytically important form of the enzyme towards acylation by substrates and alkylation by alkylating agents based on the observation that kinetic solvent deuterium isotope effects on these rate processes near the pH-optimum of activity are significantly less than the values of 2–3 commonly anticipated for general base catalysis [4,5]. This conclusion is discussed within the context of (a) the observed inverse kinetic solvent deuterium isotope effect on the rate of alkylation of papain by chloroacetate reported here and (b) the potential for inverse equilibrium solvent deuterium isotope effects on $K_{32}$ deduced from the effect of $D_2O$ on the mercaptide ion-like difference absorption spectrum of papain reported in [6].

*Abbreviation:* L, hydrogen ion or deuterium ion

## 2. Materials and methods

Chloroacetamide and chloroacetate (Eastman) were twice recrystallized from warm acetonitrile. The *N*-carbobenzoxy glycine-*p*-nitrophenyl ester was a Sigma product. Deuterium oxide, 99.8% (Bio-Rad) was distilled once under nitrogen. Papain (Worthington) was prepared by the method in [7].

Second order rate constants for alkylation of papain by chloroacetamide or chloroacetate in $H_2O$ and $D_2O$ versus pL were determined from the first order rates of loss of enzymic activity in the presence of excess alkylating agent in inactivation mixtures composed of a nitrogen saturated wide-range phosphate–borate–acetate buffer (40 mM in each buffer component) containing 5 mM EDTA under a nitrogen atmosphere. The enzyme active sites were generally 0.02 mM while the alkylating agent was 0.25–5 mM, depending on the pL. The inactivation mixtures were ionic strength adjusted using NaCl. Enzymic activity of inactivation mixtures was monitored with *N*-carbobenzoxyglycine-*p*-nitrophenyl ester by following the appearance of *para*-nitro phenoxide ion at 410 mm as a function of time. For $D_2O$ solutions, pD was determined by adding 0.4 to the pH-meter reading.

## 3. Results

Chloroacetate and chloroacetamide rapidly and irreversibly inactivate papain by alkylation of the active site sulfhydryl group. For chloroacetate, the bell-shaped pH-dependence of the inactivation rate profile can be attributed to a much larger rate constant of alkylation of neutral pH forms of the enzyme (II,III) than that for the low pH form (I) or high pH form (IV) of the enzyme [8]. The small, if not negligible, rate constant for alkylation of IV could be due to electrostatic repulsion between the active site mercaptide ion and the ionized carboxyl of chloroacetate [8]. Figure 1 shows the pL-dependence of the second order rate constants of inactivation in $H_2O$ and $D_2O$. The inactivation rate was first order in chloroacetate in both $H_2O$ and $D_2O$ at pL 6.5 over 0.2–2.0 mM. The data in the figure corresponds to the alkaline limb of the bell-shaped curve first reported in [8]. The acid limb of the curve was not determined because of the ambiguity introduced by not knowing the effect of the state of protonation of the carboxyl group of chloroacetate ($pK_a \sim 3$) on the inactivation

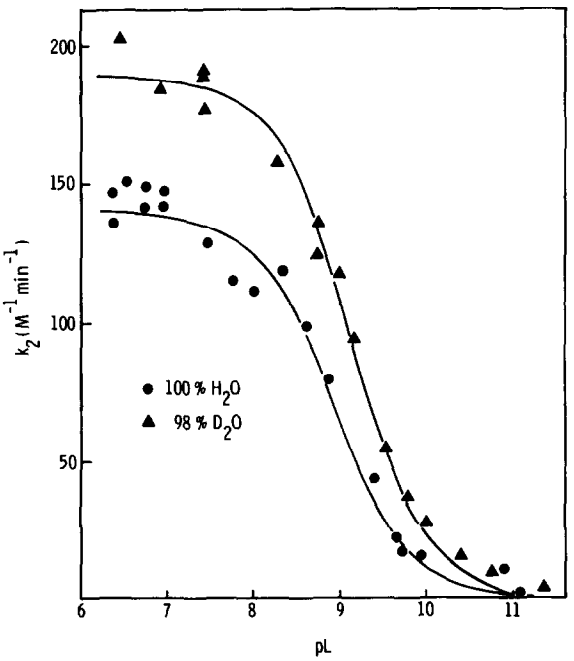



Fig.1. The pL dependence of the second order rate constants of inactivation of papain by chloroacetate in 100% $H_2O$ (•) and 98% $D_2O$ (▲) in phosphate–borate–acetate buffer (40 mM in each buffer component), EDTA = 5 mM, ionic strength 0.32 M, 25°C.

rate constant. The data could be computer fit reasonably well by eq. (1) in which $\bar{k}_2$ is the intrinsic second order rate constant:

$$k_2^{obs} = \bar{k}_2 \frac{[L^+]}{[L^+] + K_B} \quad (1)$$

for reaction of chloroacetate with the sum of forms II and III of the enzyme and $K_B$ is the apparent ionization constant controlling the conversion of II and III to much less reactive IV. The values of $\bar{k}_2$ in $D_2O$ and $H_2O$ are $189 \pm 7$ $M^{-1}$. $min^{-1}$ and $141 \pm 7$ $M^{-1}$. $min^{-1}$, respectively, corresponding to an inverse kinetic isotope effect of $0.75 \pm 0.07$. The values of $pK_B$ in $D_2O$ and $H_2O$ are $9.13 \pm 0.15$ and $8.92 \pm 0.15$, respectively, corresponding to $\Delta pK = 0.21 \pm 0.3$.

As a test of whether the inverse isotope effect observed with chloroacetate near neutrality could be due to an intrinsic isotope effect on the rate of alkylation of a mercaptide ion, the kinetic isotope effect on the rate of alkylation of the high pL-form of papain (IV) was determined from the pL-dependence of the rate of inactivation of papain by chloroacetamide in $H_2O$ and $D_2O$, fig.2. Chloroacetamide was chosen for this study since this reagent, unlike chloroacetate, reacts most rapidly with form IV of the enzyme, resulting in a sigmoidal pL dependence [9]. Approximately 85% of the experimental curve could be fit to eq. (2) in which $\bar{k}_3$ is the intrinsic second order rate constant for alkylation of the high pL form of

$$k_3^{obs} = \bar{k}_3 \frac{k_B}{k_B + [L^+]} \quad (2)$$

papain, IV. The deviation from the theoretical titration curve near neutral pL is like that observed in [8] and indicates that some form(s) of the enzyme near neutral pL reacts with chloroacetamide at a significant although much slower rate than with the high pL form [9]. This region has been studied down to $pL \sim 3$ using bromoacetamide and methylbromo acetate [5]. From the computer best fit of the data to equation 2, $pK_B$ in $H_2O$ and $pK_B$ in $D_2O$ was $8.77 \pm 0.06$ and $8.89 \pm 0.06$, respectively, corresponding to $\Delta pK_B = 0.12 \pm 0.12$. The isotope effect on the limiting value for $\bar{k}_3$ was essentially unity within experimental error, $\bar{k}_3(H_2O)/\bar{k}_3(D_2O) = 291 \pm 5$ $M^{-1}$. $min^{-1}$/ $300 \pm 7$ $M^{-1}$. $min^{-1} = 0.97 \pm 0.04$.

As an additional check on whether this was a reasonable intrinsic isotope effect to expect on the

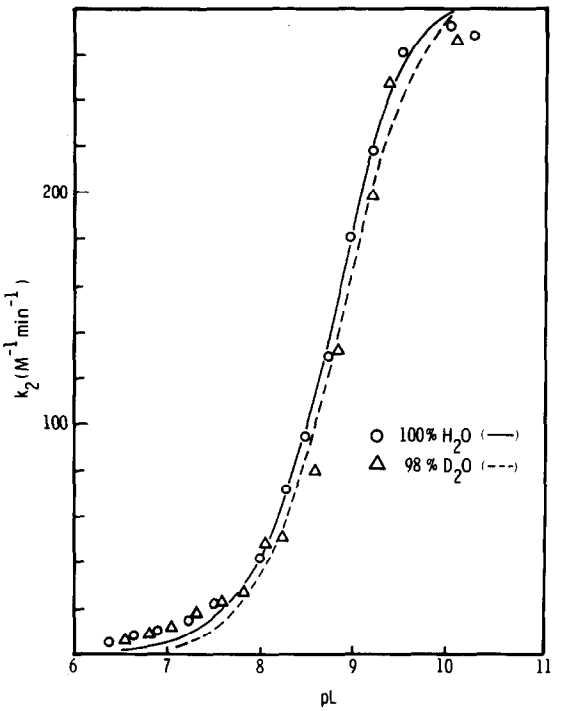



Fig.2. The pL dependence of the second order rate constants of inactivation of papain by chloroacetamide in 100% $H_2O$ (○) and 98% $D_2O$ (△) in phosphate–borate–acetate buffer (40 mM in each buffer component), EDTA = 5 mM, ionic strength 0.32 M, 25°C.

rate of alkylation of a mercaptide ion, the solvent deuterium isotope effect on the rate of alkylation of cysteine by bromoacetate was determined in 0.1 N NaOH (in $H_2O$) and 0.1 N NaOD (in $D_2O$). The second order rate constants obtained in 100% $H_2O$ and 98% $D_2O$ were $1.141 \pm 0.015\ M^{-1}.s^{-1}$ and $1.141 \pm 0.013\ M^{-1}.s^{-1}$, respectively, which corresponds to $k_{H_2O}/k_{D_2O} = 1.00 \pm 0.02$.

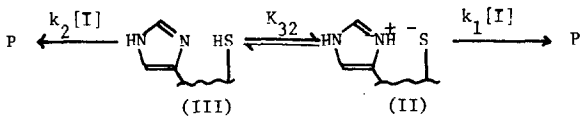
## 4. Discussion

Since alkylation of the active site sulfhydryl group of papain near the pH-optimum of activity is undoubtedly a simplier nucleophilic process than acylation by substrates, the discussion that follows focusses on the possible origins of observed kinetic solvent deuterium isotope effects on these alkylation rates reported here and elsewhere. Previously, an isotope effect near unity on the rate of alkylation by methylbromoacetate was presented as central evidence against general base catalysis, consistent with II being the reactive form of the enzyme [5]. The inverse isotope effect observed with bromoacetamide ($k(H_2O)/k(D_2O) = 0.74$) was attributed to the enhanced stability in $D_2O$ of a kinetically important hydrogen bonding interaction between the aspartate 158 peptide carbonyl oxygen atom and the amide-$NH_2$ group of alkylating agent [5]. On the other hand, the rate of alkylation of the active site sulfhydryl by chloroacetate, which is not capable of such a hydrogen bonding interaction, is also subject to a substantial inverse isotope effect, as reported here ($k_{H_2O}/k_{D_2O} = 0.75$). An alternative explanation for these isotope effects is presented here that, in our view, requires utmost consideration before any final decisions are made regarding the reactive forms of the enzyme. This is particularly true in view of the uncertainties associated with the equilibrium distribution of II and III in the active site [6].

Minimally, the observed isotope effects on the rate of alkylation of the tautomeric forms of papain are determined by eq. (3) in which superscripts H and D refer to $H_2O$ and $D_2O$ solvent, respectively:

$$\frac{k^H_{obs}}{k^D_{obs}} = \frac{k_2^H + k_1^H K_{32}^H}{k_2^D + k_1^D K_{32}^D}\left[\frac{1 + K_{32}^D}{1 + K_{32}^H}\right] \qquad (3)$$

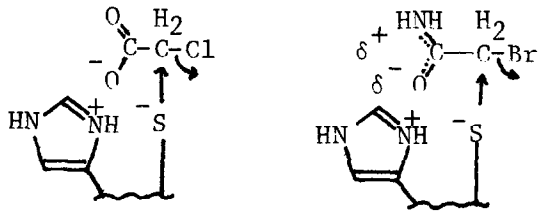
The equation was derived from the following scheme in which I is alkylating agent, P is alkylated enzyme and $K_{32} = [II]/[III]$.




The inverse isotope effects observed with chloroacetate ($k_{H_2O}/k_{D_2O} = 0.75$) and bromoacetamide ($k_{H_2O}/k_{D_2O} = 0.74$) can be accounted for provided that (a) these reagents preferentially react with II ($k_2 \simeq 0$) and (b) $K_{32}^H \simeq 2$ as suggested from the effect of solvent $D_2O$ on the mercaptide ion-like spectral properties of papain [6]. The magnitude of $K_{32}$ in $D_2O$ can be predicted from the isotopic fractionation factor of the N–H bond ($\phi = 0.97$) and S–H bond ($\phi \simeq 0.4$), $K_{23}^D = (\phi_{NH}/\phi_{SH})(2) \simeq 5$ [10]. Under these conditions eq. (3) reduces $k^H_{obs}/k^D_{obs} \simeq (k_1^H/k_1^D)(0.8)$. The isotope effect on $k_1^H/k_1^D$ is reasonably predicated to be $\simeq 1$ since there are no apparent proton transfers from the

sulfur atom during alkylation. In addition, the intrinsic isotope effect on the rate of alkylation of a free mercaptide ion is near unity as evidenced by the observed isotope effect of 1.0 ± 0.0 for alkylation of the high pL form of cysteine by bromoacetate and the observed isotope effect of 0.97 ± 0.04 for alkylation of the high pL form of papain (IV) by chloroacetamide, fig.2. Thus, the anticipated isotope effect for preferential reaction with II is 0.8, corresponding well to the observed isotope effects. This isotope effect has been reproduced in a simple chemical model system: The bell-shaped pL-dependence of the rate of alkylation of cysteine ($K_{32}^{H}$ = 2.1 [6]) by chloroacetate is subject to an inverse kinetic solvent deuterium isotope effect of ~0.8 at the top of the bell-shaped curve where the thiolate–ammonium ion and thiol–amine tautomers of cysteine predominate (unpublished).

On the other hand, reaction of chloroacetate and bromoacetamide with III cannot be rigorously excluded because of the indeterminant nature of the isotope effect on $k_2$. In the extreme case where these reagents react exclusively with III ($k_1$ = 0), eq. (3) reduces to $k_{obs}^{H}/k_{obs}^{D} \simeq (k_2^{H}/k_2^{D})$(2). From a linear approximation of isotope effects, $k_2^{H}/k_2^{D}$ could range from a maximum value of ~6, for a transition state in which the proton is ~50% transferred to the imidazole function, to a minimum value of ~0.4 for a completely product-like transition state in which the proton is completely transferred to imidazole [10]. (This is calculated from the isotopic fractionation factor of the S–H bond in the ground state ($\phi \sim 0.4$) and the isotopic fractionation factor of the N–H bond in a completely product-like transition state ($\phi = 0.97$), such that $k_2^{H}/k_2^{D} = \phi_{SH}/\phi_{NH} \sim 0.4$.) If the completely product-like transition state were to apply, $k_{obs}^{H}/k_{obs}^{D} \simeq 0.8$, the same value predicted for reaction with II. On the other hand, we suggest that preferential reaction with II is most likely based on our perception that the negatively charged carboxyl of chloroacetate and the polarizable amido function of bromoacetamide is a critical factor in determining preference:



For the comparatively less polar alkylating agent, methylbromoacetate, preference for reaction with II may be lost. Inspection of eq. (3) indicates that in this case the observed isotope effect would depend on $k_2^{H}/k_2^{D}$ and the relative magnitude of $k_1^{H}$ and $k_2^{H}$. Thus, an observed isotope effect of unity could be accounted for by several kinetic cases, one of which would be where $k_2^{H}/k_2^{D} = 1$ and $k_2^{H} = k_1^{H}$.

## References


[1] Glazer, A. N. and Smith, E. L. (1971) The Enzymes 3, 501.
[2] Husain, S. S. and Lowe, G. (1968) Biochem. J. 108, 855.
[3] Drenth, J., Jansonius, J. N., Koekoek, R., Wolthers, B. G. (1971) The Enzymes 3, 485.
[4] Polgar, L. (1973) Eur. J. Biochem. 33, 104.
[5] Polgar, L. (1979) Eur. J. Biochem. 98, 369.
[6] Creighton, D. J. and Schamp, D. J. (1980) FEBS Lett. 110, 313–318.
[7] Sluyterman, L. A. AE. and Wijdenes, J. (1970) Biochim. Biophys. Acta 200, 593.
[8] Chaiken, I. M. and Smith, E. L. (1969) J. Biol. Chem. 244, 5095.
[9] Chaiken, I. M. and Smith, E. L. (1969) J. Biol. Chem. 244, 5087.
[10] Schowen, R. L. (1972) Prog. Phys. Organ. Chem. 9, 275.